Original Article

CD4$^+$ T cell proliferation and inhibition of activation-induced cell death (AICD) in childhood asthma

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Abstract: Objective: The mechanism underlying immune inflammation and over-activation of T helper (Th) cells in childhood asthma was investigated through cell proliferation and activation-induced cell death (AICD) experiments. Methods: There were 30 children in the asthma group with an average age of 10.2 ± 3.1 years and 30 children in the normal control group with an average age of 10.5 ± 2.9 years. A cytometric bead array (CBA) was performed to detect Th1, Th2, and Th17 cytokines. After CD4$^+$ T cells were separated using immunomagnetic beads and stimulated by phytohemagglutinin (PHA) combined with anti-CD3 antibodies in vitro, cell proliferation and AICD were analyzed. Finally, mRNA expression of the apoptosis- and proliferation-related proteins Fas, Fasl, and Bcl-2 was detected using quantitative polymerase chain reaction (PCR). Results: The serum cytokine levels of the children in the asthma group significantly increased compared to those of children in the normal control group (IL-4: 9.33 ± 2.25 ng/L vs. 4.59 ± 1.77 ng/L, P = 0.012; IL-10: 3.41 ± 0.80 ng/L vs. 1.36 ± 0.41 ng/L, P = 0.027; TNF: 7.63 ± 4.09 ng/L vs. 1.88 ± 0.52 ng/L, P = 0.023). The CD4$^+$ T cell proliferation ability in the asthma group was significantly higher than that in the normal control group (OD450: 0.66 ± 0.14 vs. 0.28 ± 0.07, P<0.001), whereas the AICD rate was significantly lower than that in the normal control group (29.46 ± 5.25% vs. 60.11 ± 4.93%, P<0.001). The Fas mRNA expression in the CD4$^+$ T cells from children in the asthma group was significantly decreased compared to that from the children in the normal control group, whereas the Bcl-2 expression was significantly higher than that from children in the normal control group. These differences were both statistically significant (P<0.001). Conversely, FasL expression did not differ (P > 0.05). Conclusion: Fas expression decreased and Bcl-2 expression increased in CD4$^+$ T cells from the children in the asthma group, inhibiting AICD, and promoting proliferation of Th cells to some extent. Apoptosis inhibition and cell proliferation might result in over-activation of Th cells and aggravation of inflammatory infiltration in children with asthma.

Keywords: Childhood asthma, proliferation, Th cells, activation-induced cell death

Introduction

Childhood asthma is an immune-associated disease caused by many factors, including genetics and the environment. An immune inflammatory reaction in the respiratory tract is a primary characteristic of asthma. Chronic inflammatory reactions mediated by T cells and related cytokines are significant in the initiation and progression of asthma [1]. Through contact with the mucosal immune system (such as the respiratory tract and intestinal tract), antigens in the external environment can induce the body to produce immune tolerance, particularly T cell tolerance. This process can effectively protect the body from developing asthma [2]. Over-activation of CD4$^+$ T cells in the peripheral blood and respiratory tract is a common feature of asthma patients and the resulting rapid proliferation of T cells and secretion of many cytokines aggravates the inflammatory reaction. The exact reasons and relevant mechanisms are currently unclear but activation-induced cell death (AICD) caused by T cell activation in the body is an important mechanism underlying the clearance of overly activated T cells and the maintenance of peripheral immune tolerance. When immune tolerance balance is destroyed by external factors, the body can suffer immune injury medi-
ated by T cells [3, 4]. This study observed prolifera-
tion of CD4+ T cells and conditions of AICD
in the peripheral blood of children with asthma
to investigate their mechanisms of action in
asthmatic immune injury. These data provide
new ideas for further development of peripher-
al immune tolerance therapy.

Material and methods

Study subjects

This study included 30 children with asthma
(17 boys and 13 girls with an average age of
10.2 ± 3.1 years) who were admitted to the
hospital. They all met the diagnostic criteria
of asthma. There were 30 cases in the normal
control group, which included 15 boys and 15
girls with an average age of 10.5 ± 2.9 years.
An assessment of children with asthma accord-
ing to the disease conditions showed that there
were 6 mild cases, 15 moderate cases, and 9
severe cases. Blood was collected from all
pediatric patients without receiving any drug
treatment. In addition, patients with tumors,
blood diseases, and other autoimmune diseas-
es were excluded.

Experimental materials

The cytometric beads array (CBA) kit and T
helper 1 (Th1), Th2 and Th17 cytokine detection
reagent kits were obtained from BD (USA). Lym-
phocyte separation solution was obtained from
MP Biomedicals (China). CD4 antibody-
labeled immunomagnetic beads were obtain-
ed from Miltenyi Biotec (Germany). RPMI1640
culture medium was obtained from Gibco (Au-
stralia). Phytohemagglutinin (PHA) was obtain-
ed from Sigma (USA). The CCK-8 cell prolifera-
tion reagent kit was obtained from Yeasen
(USA). The Annexin V/PI apoptosis detection
reagent kit was obtained from Shanghai Sangon
Biotech (China).

Detection of cytokines

Serum samples from the children were sepa-
rated, and interleukin (IL)-2, IL-4, IL-6, IL-10,
tumor necrosis factor (TNF), interferon (IFN)-γ,
and IL-17A were detected using CBA technol-
ogy strictly according to the operational proce-
dures in the reagent instructions. First, the
standard samples were used for gradient dilu-
tion at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128
and 1:256. In addition, fluorescent micro-
spheres were mixed and re-suspended at room
temperature in the dark for 30 min. Next, the
relevant parameters of the flow cytometer
(Beckman FC500) were adjusted and set up to
enter the detection status. Finally, 50 µL of the
diluted standard sample was added to a stan-
dard sample tube, and 50 µL of the specimen
to be detected was added to a sample tube.
Fifty microliters of PE-labeled human Th1/Th2/
Th17 detection reagents was added to all
experimental tubes, and the tubes were incub-
ated in the dark for 3 h before being loaded
onto the machine for detection.

CD4+ T cell separation and purification

A total of 5 ml of peripheral blood was col-
lected from the children using EDTA-K2 as the
anti-coagulant. Peripheral blood mononuclear
cells (PBMCs) were separated using the lymph-
ocyte separation solution and washed with
pre-cooled phosphate-buffered saline (PBS) 3
times for future use. The above PBMCs and
anti-CD4-labeled magnetic beads were vor-
texed at 4°C, incubated for 15 min, and passed
through the column to separate the CD4+ T
cells. The cells were washed with pre-cooled
PBS 3 times. After being labeled with anti-CD4-
FITC, the purity of the CD4+ T cells was analyzed
using flow cytometry.

Cell proliferation experiments

CD4+ T cells with a purity > 95% were adjusted
to 1 × 10^5/ml using RPMI1640 complete medi-
um (10% fetal bovine serum, 50 µg/ml penicil-
lin, 50 µg/ml streptomycin, 2 mmol/L gluta-
mine, and 10 mmol/L HEPES) and cultured in
96-well plates. PHA was added to the solution
to reach a final concentration of 10 µg/ml. In
addition, PBS was used as the experimental
control in the asthma group and the normal
control group. The cells were cultured at 37°C
and 5% CO₂ for 3 d. Ten microliters of CCK8 was
added to each well 4 h before harvesting the
cells. Proliferation was observed by measuring
the absorbance at 450 nm after 4 h.

AICD experiments

The cell concentration was adjusted to 2 × 10^5/
ml using RPMI complete culture medium, and
the cells were cultured in 6-well plates. The cells
were combined with 10 µg/ml PHA and
CD4+ T cell proliferation and AICD in childhood asthma

Table 1. Expression of serum T cell-related cytokines of children in the asthma group and the normal control group (x ± s, ng/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>IL2</th>
<th>IL4</th>
<th>IL6</th>
<th>IL10</th>
<th>TNF</th>
<th>IFN-γ</th>
<th>IL17A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>2.35 ± 0.41</td>
<td>9.33 ± 2.25</td>
<td>3.65 ± 1.42</td>
<td>3.41 ± 0.80</td>
<td>7.63 ± 4.09</td>
<td>2.05 ± 1.33</td>
<td>4.38 ± 1.32</td>
</tr>
<tr>
<td>Normal control</td>
<td>1.98 ± 0.66</td>
<td>4.59 ± 1.77</td>
<td>3.10 ± 1.78</td>
<td>1.36 ± 0.41</td>
<td>1.88 ± 0.52</td>
<td>1.99 ± 1.17</td>
<td>4.06 ± 1.50</td>
</tr>
<tr>
<td>P value</td>
<td>0.159</td>
<td>0.012</td>
<td>0.358</td>
<td>0.027</td>
<td>0.023</td>
<td>0.845</td>
<td>0.479</td>
</tr>
</tbody>
</table>

Figure 1. Proliferation of CD4+ T cells from asthmatic or normal children. CD4+ T cells were isolated from asthmatic or normal children and then activated by PHA or PBS. Cell proliferation was observed by measuring the absorbance at 450 nm. P<0.001 vs. control.

Quantitative PCR

The total RNA of the CD4+ T cells was first extracted and then reverse transcribed into cDNA. The cDNA was used as the template for quantitative polymerase chain reaction (PCR) amplification using a PCR reaction reagent kit (Applied Biosystems), and β-actin was used as the internal control. 2−ΔΔCt was calculated using the formula ΔΔCt = [Ct (target gene)]-[Ct (internal control gene)] and ΔΔCt = [ΔCt (asthma group)]-[ΔCt (control group)] to reflect the expression level of the target genes in the asthma group.

Statistical methods

SPSS 18.0 software was used. All data are measurement data and are expressed as mean ± standard deviation (SD). Cytokines were analyzed using analysis of variance, and other indicators were examined using the two-sample t test. P<0.05 indicated that the difference was statistically significant.

Results

Cytokine detection results

The serum IL-4, IL-10, and TNF levels of the children in the asthma group were all significantly higher than those of the children in the normal control group, and the differences were statistically significant (P<0.05). The expression levels of IL-2, IL-6, IFN-γ and IL-17A in the asthma group did not have significant changes compared to those in the normal control group, and the differences were not statistically significant (P > 0.05, Table 1).

Cell proliferation experiment results

The results of the absorbance of cell proliferation in each group are shown in Figure 1. In the normal control group, the proliferation of CD4+ T cells was 0.19 ± 0.02 when treated with PBS and 0.28 ± 0.07 when treated with PHA, (P<0.001) whereas in the asthma group, the proliferation of CD4+ T cells treated with PBS and PHA was 0.18 ± 0.03 and 0.66 ± 0.14 (P<0.001). The proliferation ability of the CD4+ T cells after PHA stimulation in the asthma group significantly increased compared to that in the normal control group (P<0.001). After treatment with PBS, the proliferation abilities between these two groups were not significantly different.
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Cell apoptosis detection results

In the normal control group, the results were 2.78 ± 1.08 in PBS group and 60.11 ± 4.93 in PHA group (P<0.001) and in the asthma group, the results were 2.66 ± 0.67 in PBS group and PHA 29.46 ± 5.25 in the PHA group (P<0.001). After PHA activation, the cell apoptosis rate in the asthma group was significantly lower than that in the normal control group (P<0.001). The difference after PBS treatment between these two groups was not statistically significant (P > 0.05, Figure 2).

The mRNA expression of Fas, FasL, and Bcl-2 in CD4⁺ T cells from children in the asthma group and children in the normal control group

The expression of Fas mRNA in the CD4⁺ T cells of children with asthma was significantly lower than that of children in the normal control group, and Bcl-2 expression in the asthma group was significantly higher than that in the normal control group. The differences were both statistically significant (P<0.001). The expression of FasL between these two groups did not display a significant change (P > 0.05, Figure 3).

Discussion

Childhood asthma is a complicated allergic disease. The main manifestations are periodic respiratory injury, chronic inflammation of the respiratory tract, and significant lymphocyte and eosinophil infiltration in the submucosal layer of the respiratory tract [2]. Studies have indicated that Th cells play important roles in the developmental process of allergic asthma. Allergens induce the body to produce a large amount of immunoglobulin E (IgE), which can activate eosinophils, basophils, and plasma cells to release various cytokines [3]. These cytokines can promote differentiation of Th cells into Th2 cells in the body to secret Th2-related cytokines and participate in mediation of the immune inflammatory reaction. The studies of Nakajima and Takatsu [6] and Kaminuma et al. [7] showed that IL-4 is involved in the mediation of local tissue inflammatory infiltration in asthma patients and can activate eosinophils to aggravate asthma symptoms.
In addition, IL-4 can participate in IgE production through the regulation of class switching. The study by Sahid El-Radhi et al. [8] showed that the IL-4 level in children with acute-phase asthma was significantly higher than that in a mild symptom group, and IL-4 was involved in the immune status changes in children with asthma. We detected serum Th1-, Th2- and Th17-related cytokines in children with asthma using CBA technology and show that IL-4, IL-10, and TNF are all significantly increased. IL-4 and IL-10 are the major Th2-related cytokines, whereas TNF can mediate immune inflammatory responses through promotion of lymphocyte aggregation and secretion of Th2-related cytokines [9].

The inflammatory injury sites of asthma patients contain many activated lymphocytes, and the survival time of these lymphocytes is significantly prolonged [10]. Under normal conditions, the immune system can clear overly activated lymphocytes through AICD to effectively prevent immune inflammatory injury and the development of autoimmune diseases. Because inflammation responses mediated by Th cells and its related cytokines play a leading role in asthmatic patients, we separated peripheral CD4+ T cells from children to perform in vitro stimulation to observe the ability of cell proliferation and the condition of AICD. The results show that CD4+ T cells in the asthma group are significantly enhanced after PHA stimulation compared to those in the normal control group, whereas AICD is inhibited. Furthermore, the apoptosis rate was significantly decreased. Therefore, lymphocyte infiltration and the prolongation of lymphocyte survival time in children with asthma might be induced by the joint effect of defects in the AICD clearance mechanism and the significant enhancement of cell proliferation ability.

AICD is cell apoptosis mediated by death receptors. Its main pathway is to recruit the adaptor protein FAS-associated death domain (FADD) through the interaction between Fas and FasL to activate downstream apoptosis-related proteins and induce cell apoptosis [11]. Under normal conditions, the body will clear overly activated T cells through AICD. However, children with asthma have many activated CD4+ T cells in the peripheral blood and tissues. Therefore, the AICD process in children with asthma might be suppressed to some extent. Detection of Fas and FasL expression in CD4+ T cells of children with asthma using quantitative PCR showed that Fas mRNA expression in the asthma group was significantly decreased compared to that in the normal control group, and FasL expression was not significantly different. These results indicate that reduction of Fas expression might be an important reason for the suppression of AICD. A study showed that the expression of the Fas protein on the surface of T cells derived from the lungs of asthma patients significantly decreased [12] and that result was consistent with our study results. Another study showed that co-culturing IL-4 with T cells could down-regulate Fas expression on the surface of T cells and that IL-4 could induce T cells to express Bcl-2 to inhibit cell apoptosis and promote cell proliferation [13]. We found that serum IL-4 levels in children with asthma significantly increased and that expression of Bcl-2 mRNA in CD4+ T cells also increased. Therefore, the inhibition of apoptosis and enhancement of proliferation ability in the CD4+ T cells of children with asthma might have an important relationship with the increase of IL-4 levels.

In summary, Th2-related cytokines significantly increased in children with asthma, the proliferation ability of CD4+ T cells significantly increased, and AICD was inhibited. The possible mechanism might be the reduction of the Fas expression level and the increase of Bcl-2 expression in CD4+ T cells caused by the increased IL-4 expression level.
Disclosure of conflict of interest

None.

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